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The *pepR* Gene of *Lactobacillus sakei* Is Positively Regulated by Anaerobiosis at the Transcriptional Level

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***Lactobacillus sakei* is a lactic acid bacterium belonging to the natural flora of meat products. It constitutes the main flora of vacuum-packed meat and is largely used in western Europe as a starter for the manufacturing of fermented sausages. This species is able to grow both under aerobiosis and anaerobiosis. In many technological processes involving it, oxygen is scarce. The aim of this study was to identify the major proteins affected by growth under anaerobiosis. Using two-dimensional electrophoresis, we showed that one spot was 10-fold overexpressed when cells were grown under anaerobiosis. By N-terminal sequencing it was identified as a peptidase (PepR), and the *pepR* gene was cloned. Northern analysis revealed that *pepR* was expressed as a single 1.27-kb transcript induced under anaerobiosis. A mutant was constructed by single crossover in the *pepR* gene, and its growth and survival were not affected by anaerobiosis.**

Lactobacillus sakei (formerly *Lactobacillus sake*) (32) is one of the most important lactic acid bacteria (LAB) in meat technology. It is naturally present on fresh meat and becomes the predominant flora on vacuum-packed meat. It is largely used in France and western Europe as a starter for the manufacturing of fermented sausages. This species is able to produce lactic acid and also some compounds that are inhibitory towards pathogenic or spoilage bacteria. It also might be involved in aroma production. Its development during technological processes is thus very important for organoleptic and safety reasons (for a review, see reference 5). This species is facultatively heterofermentative, and it is able to grow under both aerobiosis and anaerobiosis. Nevertheless, during the main technological process in which it is involved, it must develop under hypoxic environmental conditions.

Two-dimensional electrophoresis (2-DE) is a powerful method to investigate variation of protein expression during environmental changes. It has been used to investigate changes induced by anaerobiosis in various species. The gram-negative species *Escherichia coli* (2, 22, 28), *Salmonella enterica* serovar Typhimurium (30), and *Neisseria gonorrhoeae* (7) and the gram-positive species *Bacillus subtilis* (20) have been studied by this approach. Such studies led to the identification of proteins whose expression was significantly modified by the presence or absence of oxygen. These proteins could be assigned to respiratory or carbon flux functions, and proteins subjected to catabolite repression have been shown to be also regulated by growth under anaerobiosis. In *E. coli*, analysis of the glucose starvation stimulon revealed a partial similarity to the response to a shift from aerobiosis to anaerobiosis (22). Proteins induced by acids or bases during aerobic or anaerobic growth were also analyzed by 2-DE. It appeared that some inductions were pH dependent only, but complex relationships between pH and oxygen were shown (2). In *N. gonorrhoeae*, a shift from aerobiosis to anaerobiosis resulted in the induction of outer membrane proteins (7). In *B. subtilis*, such a shift resulted in the induction of various systems for the utilization of alternative carbon sources (inositol, melibiose, and 6-phospho-α-glucosides). Mutants affected in global regulatory proteins were also analyzed, and this allowed identification of the genes whose regulation was independent from these systems (20).

The aim of this study was to identify the major protein(s) of *L. sakei* affected by growth under anaerobiosis. In this paper we show that a peptidase is 10-fold overexpressed during growth under anaerobiosis. The corresponding gene, *pepR*, was identified, and its regulation was studied.

MATERIALS AND METHODS

Strains and growth conditions. Two *L. sakei* strains were used. The wild-type strain 23K (1) and a *pepR* mutant, RV4041, were routinely grown at 30°C in MRS medium (8) or MCD medium (13) supplemented with glucose (5 g liter^{-1}) as the carbon source. When necessary, media were supplemented with 5 mg of erythromycin liter^{-1}. *E. coli* TG1 was used for cloning experiments. It was routinely grown at 37°C on Luria-Bertani medium supplemented with ampicillin (50 mg liter^{-1}) when necessary. Aerobiosis was obtained by shaking flasks at 200 rpm. Anaerobiosis was obtained either under nitrogen (12) or under carbon dioxide using the Anaerogen GasPak (Oxoid) system in BBL jars. Growth was evaluated by measuring the optical density at 600 nm (OD_{600}).

Growth and survival of the *pepR* mutant (RV4041) were tested at 30°C in MCD medium under both aerobiosis and anaerobiosis in the presence or absence of 10 μg of puromycin ml^{-1} for up to 40 h. Growth and survival after addition of 20 μg of puromycin ml^{-1} at the end of the exponential phase of growth were also monitored under the same conditions. The effect of temperature was tested by comparing the ability to grow on MRS agar supplemented or not with puromycin 10 μg ml^{-1} and by incubating plates at either 30 or 37°C under aerobiosis or anaerobiosis.

2-DE and N-terminal sequencing. Analyses were performed as previously described on 30-μg protein extracts (19). Gels were stained using silver nitrate (19). In some experiments Sypro Orange (Molecular Probes) was used. After the second dimension was run, gels were fixed for 30 min in 7.5% acetic acid–0.05% sodium dodecyl sulfate. Staining was performed by incubation for 1 h in 0.02% Sypro Orange–7.5% acetic acid. Reading was performed on a fluoroimager with excitation at a wavelength of 488 nm (Molecular Dynamics). Quantification was done by using ImageQuant software (Molecular Dynamics).

PCR amplification of the *pepR* gene and cloning of the *pepR* region. Degenerate primers 5′-TTC(T)ATGCAC(T)TGC(T)TTA(G)CC-3′ and 5′-(A,T,C,G

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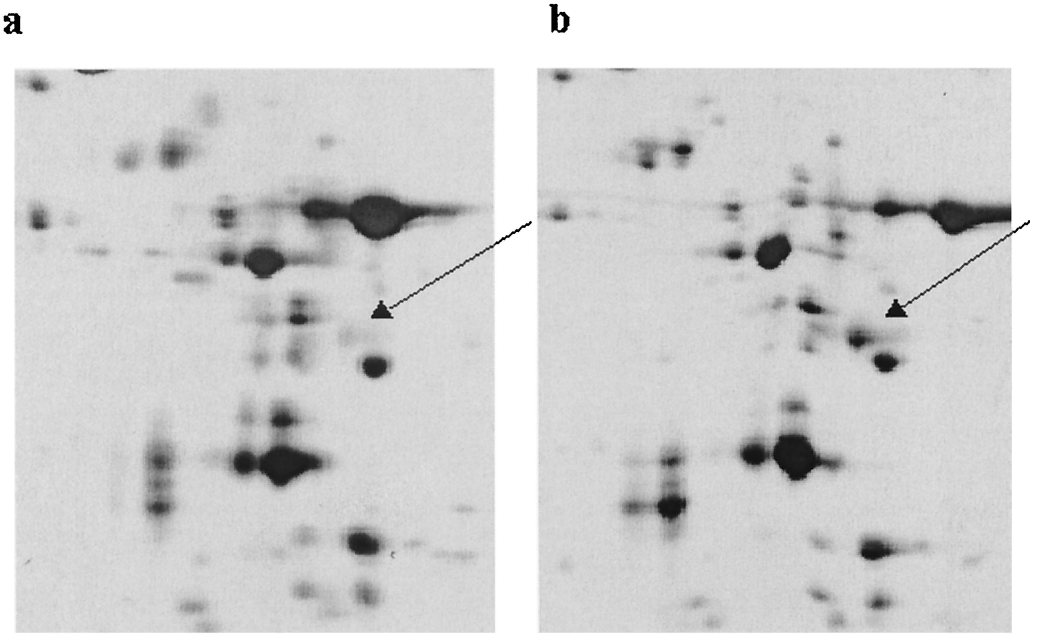

FIG. 1. Portions of two-dimensional gels showing expression of PepR of *L. sakei* under aerobiosis (a) and under anaerobiosis (b). The arrows indicate the position of PepR. Proteins were extracted from early-stationary-phase cells.

)CGG(A)TTT(C)TC(A,T,C,G)GCT(C)TC-3′ were used for the PCR amplification of a DNA fragment of strain 23K. PCR experiments were performed on a Perkin-Elmer 9600 apparatus using *Taq* DNA polymerase from Promega. Reactions were carried out in 10 μl with 1 μg of chromosomal DNA, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 2.5 μM concentration of each primer, and 1.25% formamide for 30 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min).

Cloning experiments were performed in pBluescript SK(+) (Stratagene) by standard methods (24) and as previously described (31). Southern hybridizations were performed with chemiluminescence using a kit, according to the instructions of the manufacturer (Amersham). DNA sequence reactions were done using dye terminator dideoxynucleotides and *Taq* DNA polymerase on a Perkin-Elmer 9600 apparatus, and products were separated on a Perkin-Elmer sequencing apparatus 337. DNA sequence was analyzed with the Genetics Computer Group software package from the University of Wisconsin.

Construction of a *pepR* mutant by single crossover. An internal fragment of the *pepR* gene was amplified by PCR and cloned in the integrative vector pRV300 (14), leading to plasmid pRV415. This plasmid was used to transform *L. sakei* strain 23K for erythromycin resistance as previously described (1, 14). The structure of the mutated *pepR* gene was verified by PCR.

Analysis of the *pepR* transcript. RNA preparation and Northern hybridization were performed as previously described (31) with the following modifications. Thirty micrograms of total RNAs was loaded on the gels. Labeling of the probes was performed with an enhanced chemiluminescence kit (ECL) according to the instructions of the manufacturer (Amersham).

Nucleotide sequence accession number. The GenBank accession number is AF402317.

RESULTS

Growth under aerobiosis and anaerobiosis and identification of PepR. *L. sakei* was grown both under aerobiosis and anaerobiosis in the chemically defined medium MCD. The growth rates observed were not significantly different as measured by the doubling times (81 ± 3 min under aerobiosis versus 79 ± 2 min under anaerobiosis). The final cell densities reached after 24 h of cultivation were also similar (0.75 to 0.8). Moreover, whatever the anaerobiosis conditions were (nitrogen or carbon dioxide), no growth difference could be observed. This is in accordance with previous data showing that anaerobiosis influenced mainly survival during stationary phase (4).

We compared protein expression under aerobiosis and anaerobiosis. Samples were prepared during exponential growth (OD_{600} = 0.4) and at the beginning of the stationary phase (OD_{600} = 0.7) and subjected to 2-DE. Several variations in protein pattern expression were observed. One major spot strongly affected by anaerobiosis was further studied. It had an apparent molecular weight (MW) of 34,918, and its pI was estimated to be 5.24. This spot was specifically overexpressed in cells grown under anaerobiosis and at the beginning of the stationary phase (Fig. 1). It was present but barely detectable when cells were grown under aerobiosis. In order to obtain a better quantification of these variations, gels were stained with the fluorescent dye Sypro Orange and the signal was quantified. This revealed a 10-fold overexpression in cells grown anaerobically.

The N-terminal sequence of the spot was determined after transfer on a polyvinylidene difluoride membrane. The obtained sequence, MKQGTTILT, was 100% identical to the N-terminal sequence of PepR of *Lactobacillus rhamnosus*, a peptidase with Pro-X specificity encoded by the *pepR* gene (34). A high identity score (66%) with N-terminal sequence of the *Lactobacillus helveticus* PepR was also observed (33). Furthermore, a peptidase in *Lactobacillus curvatus*, a species closely related to *L. sakei*, has been purified and the 20 N-terminal amino acids have been determined (18). The N-terminal sequence of PepR of *L. sakei* differs in only one amino acid from this sequence. Also, the estimated MW and pI for this *L. sakei* protein were in accordance with those of *L. rhamnosus* and *L. helveticus* PepR. Moreover, the estimated MW was also in accordance with the MW of the purified peptidase of *L. cur-*

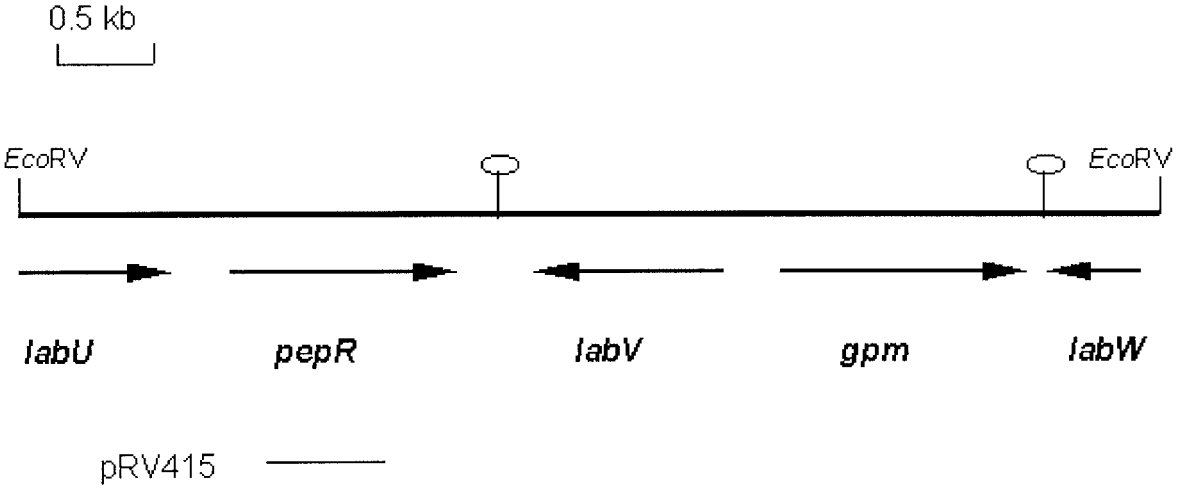

FIG. 2. Schematic representation of the *pepR* encoding region. The insert of plasmid pRV415 and putative transcription terminators are shown.

vatus (18). We therefore concluded that this protein should be a PepR prolinase encoded by a *pepR* gene.

Cloning and analysis of the *pepR* gene cluster. Comparison of the *L. helveticus* and *L. rhamnosus* PepR proteins revealed conserved motifs. One of them, GGHHHM, located at positions 267 to 274, as well as the N-terminal sequence of PepR of *L. sakei* were used to design degenerate primers. A 829-bp fragment was PCR amplified, and the sequence revealed a protein 69 to 75% identical to other PepR proteins known so far and with lesser identities to various peptidases. The PCR fragment was used as a probe in Southern experiments on chromosomal DNA of *L. sakei* 23K digested with various restriction enzymes. This allowed to identify a 3.3-kb *Eco*RV fragment hybridizing with the *pepR* probe. An *Eco*RV genomic library was constructed in *E. coli* TG1 by the use of pBluescript SK(+) and screened with the *pepR* probe. This allowed us to obtain a fragment containing the *pepR* gene, leading to plasmid pRV416. The gene organization of the fragment is shown in Fig. 2. Five open reading frames (ORFs) were found in this region. PepR of *L. sakei* is encoded by a 903-bp ORF. The protein is 75.5% identical to PepR of *L. rhamnosus*. The motif GQSWGG, which corresponds to the active site of prolyl oligopeptidases (23) found in PepR of *L. rhamnosus* and *L. helveticus* and also in PepI of *Lactobacillus delbrueckii* and *L. helveticus* (6), is also present in *L. sakei*. The calculated MW is 34,240.28 and the estimated pI is 4.85, in agreement with the experimental values determined on 2-DE gels (see above). A putative ribosome binding site was found 10 nucleotides upstream from the start codon. The *pepR* gene is followed by an inverted repeat structure ($\Delta G^\circ = -24$ kcal) located 45 nucleotides downstream from the stop codon and resembling a transcription terminator. A 457-bp ORF (*labV*) was observed starting 338 bp downstream from the stop codon of *pepR*. *labV* is 25% identical to *laaB*, an hypothetical transcriptional regulator of *L. sakei* (accession number AF115391). It also shares 25% identity with various other transcriptional regulators in different species. A 656-bp ORF (*gpm*) starts 262 nucleotides downstream from *labV* and is 30 to 40% identical to various prokaryotic and eukaryotic 2-phosphoglyceromutases (9). In its N-terminal part the encoded protein has the phosphoglycerate mutase family phosphohistidine signature. At 50 nucleotides downstream from the stop codon of *gpm*, a 291-bp partial ORF showing 25% identity to *rbsR* of *L. sakei* (accession number AF115391) and various other transcriptional regulators was observed.

We found the 3′ end of a partial ORF (*labU*) 51 nucleotides upstream from *pepR*. It shows 25% identity to several proteins of the potassium transporter family (TrkA). Furthermore, this ORF is highly similar (53% over 58 amino acid residues) to the ORF located upstream from *pepR* in *L. rhamnosus* (accession number AJ003247). It thus seems that the organization of the upstream region of *pepR* in *L. sakei* is similar to that in *L. rhamnosus* (34). However, the gene organization downstream from *pepR* differs, and we did not find in *L. sakei* any ORF identical to ORF2 described for *L. rhamnosus*. This organization is different from that of *L. helveticus*, in which *pepR* is located downstream and in the opposite direction of an ABC transporter gene (33).

Transcriptional analysis. Total RNAs were prepared from cultures grown under aerobiosis and anaerobiosis at the beginning of the stationary phase. Expression of the *pepR* transcript under these two conditions was measured by Northern analysis (Fig. 3). Using a 830-bp *pepR* probe, we determined that the *pepR* gene is expressed as a single 1.27-kb transcript and is 20-fold overexpressed under anaerobiosis. This is in accordance with the estimated size of the *pepR* gene. From the results of DNA sequence analysis, a transcription terminator might exist 45 bp downstream from the stop codon of *pepR*. A promoter should thus be present 1.27 kb upstream from the terminator, i.e., in *labU*. However no obvious promoter sequences were observed in this region, based on the consensus hexamers TTGACA and TATAAT (17), and no TG motif could be observed (36).

Similarly, in *L. helveticus*, the 0.9-kb ORF encoding *pepR* was expressed as a single 1.25-kb transcript during the exponential phase of growth (33). In *L. rhamnosus* the *pepR* gene is also 0.9 kb long. Two transcripts of 1 and 1.5 kb were observed, the latter corresponding to both *pepR* and an upstream ORF

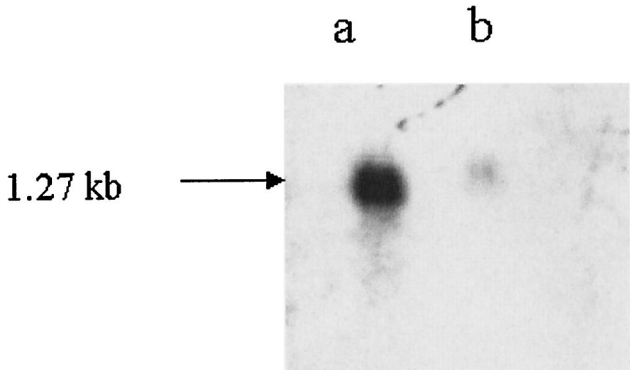

FIG. 3. Transcriptional analysis of *pepR* of *L. sakei* under anaerobiosis (lane a) and under aerobiosis (lane b).

named ORF2 of unknown function. These transcripts were overexpressed at the end of the exponential phase (34).

Construction of a *pepR* mutant and phenotypic analysis. In order to investigate the involvement of PepR in anaerobic metabolism of *L. sakei*, a *pepR* mutant was constructed by single crossover. A 660-bp internal fragment of the *pepR* gene was PCR amplified and cloned in the pRV300 integrative vector developed for *L. sakei*, leading to pRV415. A *pepR*-negative strain (RV4041) was then obtained. This strain was analyzed by 2-DE. The PepR spot was no longer detected (not shown). No other change was observed in the protein pattern of this mutant strain in comparison with the wild-type strain (data non shown). The behavior of the mutant was analyzed under both aerobiosis and anaerobiosis. No difference was observed between the mutant and the wild type regarding growth rates and survival. *pepR* mutants of *L. helveticus* (29) and *L. rhamnosus* (34) have also been constructed. Their growth in MRS or milk were identical to those of the wild type.

We also investigated possible involvement of PepR in protein repair mechanisms by studying growth and survival in the presence of puromycin. This compound is a tRNA analog which leads to the production of misfolded puromycyl-containing peptides. In *L. lactis*, mutants affected in protein repair mechanisms, such as *clpP* mutants, are affected in their growth in the presence of 10 μg of puromycin ml^{-1} (10). In *L. sakei* the *pepR* mutant did not show any clear modification of growth characteristics in the presence of puromycin at concentrations ranging from 1 to 20 μg ml^{-1} (data not shown). It also did not show any difference from the wild-type strain in behavior at 37°C.

DISCUSSION

In this study we identified a peptidase (PepR) of *L. sakei* which is 10-fold overexpressed when cells are grown under anaerobiosis. We cloned the gene and its surrounding region. We show here that the overexpression of PepR is due to a transcriptional regulation of the *pepR* gene. This transcriptional regulation (20-fold) is consistent with the 10-fold overexpression of the protein as measured in 2-DE gels. No obvious transcription promoter could be observed, and no classical regulatory motif was detected upstream from *pepR*. However, the transcriptional promoters of several LAB, including peptidase-encoding genes, showed great variability. Moreover, no information is yet available on sigma factors in *L. sakei*.

PepR has been identified only in species of the genus *Lactobacillus.* It has been purified from *L. helveticus* (29, 33), *L. rhamnosus* (34), and *L. curvatus* (18). The encoding genes have been cloned from *L. helveticus* and *L. rhamnosus*, but *pepR* is absent in the *Lactococcus lactis* IL-1403 genome (3). PepR seems to be highly conserved in lactobacilli (65 to 75% identity).

PepR cleaves Pro-X dipeptides. A putative role in flavor has been proposed for this enzyme, since proline-containing peptides have a bitter taste (see reference 34 and references therein). Mutants with mutations in these genes have been constructed but were not tested for such properties. These *pepR* mutants did not show a different growth rate in MRS medium or in milk compared to the wild type. Similarly, the *L. sakei pepR* mutant also did not show any difference in growth rate or survival ability, under neither aerobiosis nor anaerobiosis. The physiological role of PepR is thus still unknown. Nevertheless, to our knowledge, the *pepR* transcriptional regulation by anaerobiosis observed in *L. sakei* is the first reported for a peptidase of an LAB. Such an induction has been described only for a peptidase of *S. enterica* serovar Typhimurium (15). In this species the *pepT* gene, encoding a tripeptidase, has been shown to be 30-fold induced at the transcriptional level. This induction was mediated by cyclic AMP receptor protein-FNR. However, in the 5′ region of *pepR* in *L. sakei*, we could not detect a putative Fnr binding site which could explain the transcriptional regulation by anaerobiosis.

Information on regulation of expression of LAB peptidases is scarce. A global analysis of transcriptional regulation of the proteolytic system of *Lactococcus lactis* was recently done by using promoter fusions with reporter genes (11). Most of the genes were shown to be regulated at the transcriptional level by nitrogen source. In *L. rhamnosus*, *pepX*, encoding an X-prolyl dipeptidyl aminopeptidase, has been shown to belong to the *glnA glnR* operon encoding glutamine synthetase (35). This peptidase was revealed to be regulated differently from other LAB peptidases. Under some conditions, a 7-kb transcript encompassing *glnA* and *pepX* was synthesized from the *glnR* promoter. This transcript was then processed by an RNase. This revealed that both proteolytic and biosynthetic enzymes could be expressed through the same mRNA (35). Recently, six *L. helveticus* peptidase-encoding genes, including *pepR*, were expressed in *Lactococcus lactis* under the control of their own promoters (16). *pepD* and *pepR* were the only two peptidases for which no expression in *Lactococcus lactis* was obtained. This expression could be obtained only under the control of the *nisA* promoter. It thus seems that their promoters were not recognized in *Lactococcus lactis*. The anaerobic regulation found for *pepR* of *L. sakei* illustrates the diversity in the regulation of peptidase expression in LAB species.

We do not yet possess knowledge on the whole proteolytic system in *L. sakei.* Only four peptidases have been purified and studied in this species: a general aminopeptidase with broad specificity (25), a dipeptidase with main specificity towards Ala-X peptides and neutral amino acids (21), an X-prolyl dipeptidyl aminotransferase (27), and a tripeptidase with broad specificity against di- or tripeptides (26). Nevertheless, their involvement in growth and physiology or their possible impact

on meat technology is not established. Furthermore, a partial ORF encoding a putative dipeptidase has also been reported (GenBank accession number X98238).

Most studies have been focused on the role of peptidases in providing LAB with amino acids. Besides this role, it is clear that peptidases might also be involved in regulatory mechanisms and in breakdown and turnover of proteins. The transcriptional regulation of *pepR* by anaerobiosis is a new insight in the regulation of expression of LAB peptidases. This study also constitutes the first report of a correlation between transcriptional and proteomic studies in expression of LAB peptidases. As we could not observe any phenotype for the *pepR* mutant, we cannot exclude the possibility that PepR has a regulatory role in the activity or function of proteins that are not essential for growth or survival or that whatever is the role of PepR, its function is also encoded by another gene.

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